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A Validated UPLC-PDA Method for Simultaneous Determination of 3 Biologically Active Isoflavans in *Trigonella stellata* Extract

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Abstract

In this study, an ultra-performance liquid chromatography (UPLC)/photodiode array method was developed for the simultaneous determination of trigonellan glucoside (**1**), isotrigonellan (**2**), and methoxy-isotrigonellan (**3**) in *Trigonella stellata* extract using an external standard method. The extract was prepared using a standardized method by maceration of the dried plant material in ethanol. The 3 isoflavans (**1**-**3**) were separated on an Acquity UPLC C18 column using gradient elution with a mobile phase consisting of 0.1% (v/v) formic acid aqueous solution and 0.1% (v/v) formic acid in acetonitrile, and ultraviolet detection. The method provides a linear correlation for all analytes over the investigated ranges with all correlation coefficients greater than 0.998. The validated lower limits of quantitation were 53, 127, and 5 μg/mL for isoflavans **1**, **2**, and **3**, respectively. Intraday and interday precisions (percent relative SD [RSD%]) were less than 8.3% and accuracy (RE%) ranged from 90% to 100%. The method's capability to remain unaffected by small, but deliberate variations in method parameters (method's reliability during normal usage) described by the robustness showed RSD% less than 4.6% measured by varying 3 different parameters. The validated method was successfully applied to simultaneously determine the concentration of the 3 new isoflavans having anti-inflammatory and antidiabetic activities. The results revealed that the validated method can be used for quality control of herbal preparations containing these or similar isoflavans that are marketed for the prevention of inflammation and as antidiabetics.

Keywords

UPLC, isoflavans, plant extract, method validation, quantitative determination

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Phytochemical investigation of the ethyl acetate and methylene chloride fractions of the ethanolic extract of *Trigonella stellata* Forssk. resulted in the isolation of 3 new isoflavans (**1**-**3**).[1] Trigonellan glucoside, (3*R*,4*S*)−4,2',4'-trihydroxy-4'-glucopyranosyl-7-methoxyisoflavan, (**1**), was the major compound isolated from the ethyl acetate fraction. Isotrigonellan, (3*S*,4*R*)-4,2',4'-trihydroxy-7-methoxyisoflavan (**2**) and methoxy-isotrigonellan, (2*S*,3*S*,4*R*)-4,2',4'-trihydroxy-2,7-dimethoxyisoflavan (**3**), were isolated from the methylene chloride fraction (Figure 1). Isoflavans, as phytoestrogens, have diverse biological activities by having the same beneficial effects as estradiol. Phytoestrogenic effects include preventing atherosclerosis,[2] hypolipidemic effects,[3] and estrogen-like activity in the modulation of vascular injury, which is strongly attributed to decreasing risk of death from cardiovascular diseases.[4] Also phytoestrogenic compounds regulate inflammatory responses.[5]

Peroxisome proliferator-activated receptor (PPAR) activation by the isoflavans **1**-**3** was tested by our research team. Isoflavan **1** was able to activate both α and β receptors, and both isoflavans **2** and **3** activated the PPARα receptor only.[1] Moreover, isoflavans **2** and **3** have shown promising in vitro

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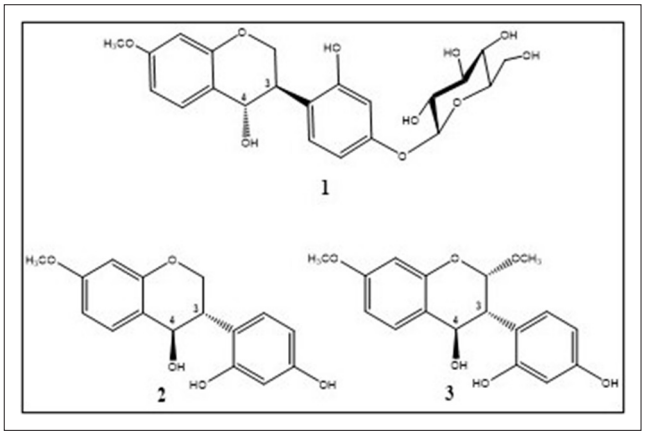

Figure 1. Chemical structure of isoflavan **1**-**3**.

anti-inflammatory activity by their ability to inhibit inducible nitric oxide synthetase (iNOS) in the mouse macrophage cell line as well as inhibiting nuclear factor kappa-B (NF-κB) activation in the human chondrosarcoma cell line (unpublished data). These results indicate that the isolated isoflavans may serve as lead compounds in the design of new agents for preventing and treating diabetes and inflammatory diseases.

Literature survey revealed that many methods have been developed for the assay of isoflavonoids in different matrices such as legume extract and biological fluids, using high-performance liquid chromatography (HPLC)[6] and UPLC-tandem mass spectroscopy (MS).[7] No method was reported for the assay of 4-hydroxyflavans.

UPLC has been used recently in the quantitation of compounds in complex matrices, especially those in low concentration, and with a high degree of accuracy. The resolution power of the UPLC makes it highly suitable for the analysis of plant extracts with complex chemical make-up.[8,9] The use of photodiode array (PDA) in conjunction with UPLC provides a high degree of accuracy in identifying the tested compound(s) in the complex matrices of plant extracts by using both the retention time and ultraviolet (UV) spectrum of each peak. The PDA detector maximizes the reliability of the method by its ability to establish peak purity.

Due to the aforementioned important biological activities, we proceeded to design and validate a method for the quantitative determination of the isoflavans **1**, **2**, and **3** in *T. stellata* alcohol extract that could be applicable to the analysis of dietary supplements containing these and similar isoflavans.

Results and Discussion

Method Development

Optimization of the conditions. The system suitability test was used to ensure that the UPLC system and procedures are adequate for the analysis performed and is an integral part of many analytical procedures. During the system suitability test, relative SD (RSD) of the peak areas was ≤2.0% in all cases, the number of theoretical plates per column was more than 3000, the USP (United States Pharmacopeia) tailing factor was ≤2.0,[10] and the resolution factor was >1.5.

Optimization of sample preparation. Different mobile phases and columns were tested to enhance the separation and shape of the peaks of the 3 isoflavans to be analyzed. Methanol, acetonitrile, and 100% of water were tested as potential mobile phases. Both Acquity UPLC C_{18} (50 mm × 2.1 mm, 1.7 μm) and CORTECS UPLC C_{18} columns (75 mm × 2.1 mm, 1.6 μm) were evaluated as stationary phases. Based on the shape of the peaks and the resolution between them, acetonitrile and 0.1% formic acid and Acquity UPLC C_{18} column were found to be the optimal mobile and stationary phases. The column temperature was 55°C, and the flow was set at 0.5 mL/min. A representative PDA contour plot is depicted in Figure 2.

Method Validation

Selectivity. The PDA contour plot (Figure 2) obtained from a representative sample showed that there is no significant interference in the chromatogram. Also, the calculated selectivity parameter having a value >1 shows the presence of a selective baseline separation between the 3 isoflavans and other matrix components.

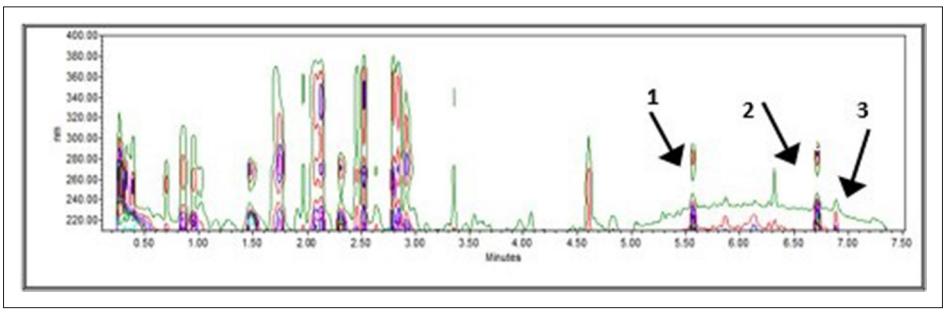

Figure 2. Ultra-performance liquid chromatography-photodiode array contour plot for the *Trigonella stellata* extract showing the separation between components and their respective retention time in minutes.

Table 1. Linear Regression Data for the Calibration Curves of the 3 Isoflavans (**1-3**).

	1	**2**	**3**
Linearity range (mg/mL)	0.01-1	0.01-1	0.01-1
Intercept (*a*)	9466.5	120 108.5	10 056.0
Slope (*b*)	3364320.9	7048151.0	17275166.0
Correlation coefficient (*r*)	0.9992	0.9981	0.9999
S_a standard deviation of X	41 075.0	178 122.2	18 845.3
S_b standard deviation of Y	17 871.3	89 508.9	8738.6
Limit of detection (mg/mL)	0.018	0.042	0.002
Limit of quantitation (mg/mL)	0.053	0.127	0.005

Linearity, limit of detection, and limit of quantitation. The calibration curve of standard isoflavan **1** showed linearity over the range 0.005-1 mg/mL, standard isoflavans **2** and **3** were linear from 0.01 to 1 mg/mL. The typical calibration curves were as follows: isoflavan **1** [Y = 9466.5 + 3364320.94 X (r = 0.9992, n = 7); isoflavan **2**, Y = 120108.45 + 7048151.04 X (r = 0.9981, n = 5); isoflavan **3**, Y = 10055.95 + 17275166.02 X (r = 0.9999, n = 5)]. Y is the peak area and X is the concentration in mg/mL (Table 1).

The limit of detection (LOD) values were 0.018, 0.042, and 0.002 mg/mL for isoflavans **1**, **2**, and **3**, respectively. The limit of quantitation (LOQ) values were 0.053, 0.127, and 0.005 mg/mL for isoflavans **1**, **2**, and **3**, respectively (Table 1).

Accuracy and precision. The precision for isoflavan **1** showed an RSD for repeatability (intraday precision) for day 1 was 3.18%, and the intermediate (interday) precision was 7.11%. For isoflavan **2**, the RSD for repeatability (intraday precision) for day 2 was 2.04%, and the intermediate (interday) precision was 2.28%. For isoflavan **3**, the RSD for repeatability (intraday precision) for day 1 was 3.07%, and the intermediate (interday) precision was 8.23%. The RSD% of the precision for the 3 isoflavans was less than 8.3% indicating that the proposed UPLC method is a rugged (reliable) quantitation method.

In testing, the accuracy in the assay of isoflavan **1**, the mean of the percent recovery for the mix I, II, and III was 97.53 ± 2.3 and RSD% = 2.31. For isoflavan **2**, the mean of the percent recovery for the mix I', II', and III' was 93.58 ± 3.1 and RSD% = 3.32. For isoflavan **3**, the mean of the percent recovery for the mix I", II", and III" was 93.95 ± 1.4 and RSD% = 1.53.

Intraday/interday precisions and accuracy were determined for the 3 tested isoflavans using the extract. The results, shown in Table 2, demonstrated that the precision showed RSD% ≤8.3, and accuracy showed RSD% ≤3.4. All the results were within the acceptance range of ±15%.[6,10]

Robustness. A slight variation of the level of the flow rate during the analysis of isoflavans **1**, **2**, and **3** resulted in only a small variation in the percent recovery with an RSD of less than 4.6%. With a slight variation in the column temperature, the RSD for the 3 isoflavans was less than 4.1%, while variations in the mobile phase composition, showed RSD of less than 2.7%. This indicates that the proposed UPLC method for the determination of isoflavans **1**, **2**, and **3** in *T. stellata* extract is a robust analytical method.

Taken together, the above results showed that a sensitive, reproducible, and robust method for the analysis of isoflavans in a complex sample matrix has been developed and validated for use in the analysis of isoflavans in herbal extracts.

The current UPLC quantitative assay has shown that *T. stellata* ethanolic extract contains 0.165%, 0.0955%, and 0.00713% of isoflavans **1**, **2**, and **3**, respectively.

Experimental

Chemicals

Ethanol was used for extraction while dimethyl sulfoxide (DMSO) was used for the preparation of samples and standard solutions. Water, acetonitrile, and formic acid used in the solvent system of the UPLC method were HPLC analytical grade and purchased from Sigma-Aldrich. Reference standards for isoflavans (**1-3**) were isolated from *T. stellata* alcoholic extract and were identified by 1-dimensional, 2-dimensional-nuclear magnetic resonance, UV, and circular dichroism experiments.[1]

Table 2. Summary of the 3 Isoflavans (**1-3**) Concentrations, Precision, Accuracy and Recovery in *Trigonella stellata* Extract.

Isoflavan	Concentration (mg/mL)	Intraday (RSD%)	Interday (RSD%)	Accuracy (RSD%)	Recovery (% mean ± SD)
1	0.331	3.2	7.11	2.31	97.53 ± 2. 3
2	0.191	2.04	2.28	3.32	93.58 ± 3.1
3	0.014	3.07	8.23	1.53	93.95 ± 1.4

RSD, relative SD.

Table 3. Robustness Results for the 3 Isoflavans (**1**-**3**).

	Flow rate		Colum temperature		% Mobile phase components	
Isoflavan	RSD	Mean% recovery	RSD	Mean% recovery	RSD	Mean% recovery
1	3.51	100.1	3.92	97.0	2.66	101.3
2	3.76	96.01	4.09	97.0	0.82	95.8
3	4.50	107.2	1.19	104.2	2.01	105.2

RSD, relative SD.

Instruments and Conditions

UPLC-electrospray ionization-MS conditions. Analyses were carried out using the Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) that consisted of a binary solvent manager, sample manager, heated column compartment, PDA detector, ESI mass detector (QDa), and a cooling autosampler. The instrument was controlled by Waters Empower 3 software. Chromatographic separations were performed on an Acquity UPLC C_{18} column (50 mm × 2.1 mm, 1.7 µm) that was maintained at 55°C. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in acetonitrile with the following gradient elution program: started at 90% A and 10% B rising to 80% A and 20% B from 0 to 4 min; then to 60% A and 40% B in 2 minutes; reaching 50% A and 50% B; then to 100% acetonitrile for 5 minutes. The system was then restored to the initial conditions in 3 minutes for a total run time of 20 minutes. The flow rate was maintained at 0.5 mL/min and, using the partial loop mode, the injection volume was 2 µL. UV spectra were recorded from 210 to 400 nm, and the quantitation wavelength was set at 284 nm.

Preparation of T. stellata *Extract*

The dried *T. stellata* herb was powdered to homogenous particle size using a coffee grinder. About 500 g were exhaustively extracted using 2 L of ethyl alcohol twice, for 2 days per extraction. The filtrate was collected, and the solvent was evaporated under reduced pressure using a rotary evaporator (BUCHI, Rotavapor R200), producing the crude extract (36 g). One gram of the crude extract was accurately weighed, quantitatively transferred into a 5 mL volumetric flask, dissolved in DMSO, and the volume was adjusted with the same solvent.

Preparation of the Standard Solutions

The stock solutions of isoflavans **1**, **2**, and **3** (trigonellan glucoside, isotrigonellan, and methoxy-isotrigonellan) were prepared by accurately weighing 2 mg of each isoflavan and quantitatively transferred into a 2 mL volumetric flask, dissolved in DMSO, and the volume was adjusted with the same solvent to give 1 mg/mL stock solution.

Method Validation

The method was validated in accordance with the Guidelines of the International Conference of Harmonization (ICH)[11] for validation of analytical procedures used for drugs and drug products and USP validation of compendial procedures.[12]

Selectivity. Selectivity was measured by comparing the peaks of the 3 isoflavans from 6 injections of the extract to that in an extract spiked with the 3 isoflavans. The quantitative analyses were carried out using UV/PDA (284 nm) for all 3 isoflavans. The selectivity of the method for each peak was further checked by comparing the λ_{max} of the UV spectrum for each peak with the spectrum of each standard compound peak appearing at the same retention time. No other components or contaminants in the plant extract were detected at the wavelength for the retention time window of the 3 isoflavans that caused any distortion of the compounds' spectra. Also, the selectivity index was calculated between isoflavan **1** and **2** and between isoflavan **2** and **3** using Empower 3 software.

Linearity. The calibration standards were prepared by serial dilution of the standard solution with DMSO to obtain 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mg/mL of isoflavan **1**; 0.01, 0.05, 0.1, 0.5, 1

Table 4. Summary of System Suitability.

	Retention time	Peak area	Capacity factor	Plate count (n)	Plate count/meter (N)	Tailing factor (*T*)
Mean	5.56	3 141 266	15.32	209 863.9	114 161.6	1.17
SD	0.02	39 309.9	0.07	-	-	0.02
RSD%	0.34	1.37	0.48	-	-	1.46

RSD, relative SD.

mg/mL for isoflavan **2**; and 0.01, 0.05, 0.1, 0.25, 1 mg/mL for isoflavan **3**. Calibration curves were established using a minimum of 5 analyte concentrations of the standard applied in duplicate and were constructed from the peak area of each analyte versus concentration. A least-squares linear regression method was used to determine the slope, intercept, and correlation coefficient of the linear regression equation. The results are shown in Table 1.

For routine analysis, a 3-point calibration curve within this range, produced by injecting duplicates of 0.05, 0.1, and 1.0 mg/mL of each standard was used with each run of the samples' solutions.

LOD and LOQ. The lower LOD is the lowest concentration of the analyte in the sample that can be detected but not necessarily quantitated within acceptable limits (±15%), under the stated experimental conditions, while the lower LOQ is the lowest concentration of the analyte that can be quantitated within acceptable limits of accuracy and precision.

According to the ICH guidelines, the detection limit (DL) may be expressed as, DL = 3.3 σ/S. While the quantitation limit (QL) may be expressed as QL = 10 σ/S. Where σ = the SD of the response and *S* = the slope of the calibration curve.

The slope *S* is estimated from the calibration curve. The estimate of σ was carried out based on the calibration curves, where the standard deviations of y-intercepts of the regression lines were used as the SD.

LOD and LOQ were experimentally verified by diluting known concentrations of the isoflavan standard solutions and measuring signal-to-noise ratio ensuring that it is 3.3:1 for the LOD and 10:1 for the LOQ. The results are listed in Table 1.

Precision. The precision may be calculated as repeatability and/or intermediate precision. Intermediate precision was performed by 6 times a day repeated analysis of the standard solution on different days.

Precision was determined by 6 independent determinations at 100% of the test concentration per day and for 2 days to determine intraday and interday precision with working calibration curves repeated daily.[11] RSD% was calculated. The experiment was repeated by the use of the same equipment, the same analytical procedure in the same laboratory, and on the same instrument. The results are shown in Table 2.

Accuracy and recovery. The accuracy of the method was validated by a standard addition analysis. This involves applying the analytical procedure to spiked mixtures of the sample to which known quantities of the standard have been added to the test solution. Thus, the sample solutions were spiked with 3 different known concentrations of the 3 standard isoflavans.

Three 100 µL aliquots of the sample solution (containing 0.33 mg/mL of isoflavan **1**) were each mixed with standard **1** of concentration 0.1, 0.25, and 0.5 mg/mL to give mix I, mix II, and mix III, respectively. Similarly, for measuring accuracy for the determination of isoflavan **2**, sample solutions were spiked with 0.05, 0.1, and 0.25 mg/mL of standard **2** separately to give mix I', mix II', and mix III', respectively. For testing the accuracy for determination of isoflavan **3**, spiking of the samples was carried out with 0.01, 0.05, and 0.25 mg/mL giving mix I", mix II", and mix III".

The original and the 3 fortified sample solutions for each compound were then analyzed on the same day in duplicates. The differences between the calculated and the found concentrations were determined to evaluate the accuracy of the method.

The percentage recovery for each solution, the mean percent recovery, SD, and RSD% for each set are given in Table 2.

Robustness. ICH defines robustness as a measure of the method's capability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Small changes in the flow rate of the mobile phase were varied in the range of ±10%. The percentage of the mobile phase solvents were varied in the range of ±2% in each solvent along with the gradient system. The column temperature was also changed by ±2°C from the controlled column temperature (55°C) used in the assay method. Robustness of the method was carried out by repeating the test procedure on the test solution and the calibration standards and changing each condition one at a time, with each condition done in triplicate. The results are presented in Table 3.

Evaluating system suitability. The standard solution of isoflavan **1** was used in system suitability testing for measuring capacity factor (*K'*), the tailing factor, and the number of theoretical plates. These measurements were carried out by injecting the standard solution 6 times and calculating the RSD% for the retention time, peak area response, capacity factor, plate count, and the tailing factor of the isoflavan **1** peak. The results are recorded in Table 4.

Declaration of Conflicting Interests

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References

1. Shams Eldin SM, Radwan MM, Wanas AS, et al. Bioactivity-guided isolation of potential antidiabetic and antihyperlipidemic compounds from *Trigonella stellate*. *J Nat Prod.* 2018;8(5):1154-1161.
2. Rosenblat M, Belinky P, Vaya J, et al. Macrophage enrichment with the isoflavan glabridin inhibits NADPH oxidase-induced cell-mediated oxidation of low density lipoprotein. A possible

role for protein kinase C. *J Biol Chem*. 1999;274(20):13790-13799. doi:10.1074/jbc.274.20.13790

3. Vaya J, Mahmood S, Goldblum A, et al. Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry*. 2003;62(1):89-99. doi:10.1016/S0031-9422(02)00445-4
4. Somjen D, Knoll E, Vaya J, Stern N, Tamir S. Estrogen-like activity of licorice root constituents: glabridin and glabrene, in vascular tissues in vitro and in vivo. *J Steroid Biochem Mol Biol*. 2004;91(3):147-155. doi:10.1016/j.jsbmb.2004.04.003
5. Karpuzoglu E, Ahmed SA. Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: implications for immunity, autoimmune diseases, and apoptosis. *Nitric Oxide*. 2006;15(3):177-186. doi:10.1016/j.niox.2006.03.009
6. Grippo AA, Xie Y, Rougeau BL, Wyatt WV. Analysis of phytoestrogens by high performance liquid chromatography. *J Arkansas Academy of Sci*. 1999;53(1):61-66.
7. Wei L, Wang X, Zhang P, et al. An UPLC-MS/MS method for simultaneous quantitation of two coumarins and two flavonoids in rat plasma and its application to a pharmacokinetic study of *Wikstroemia indica* extract. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2016;1008:139-145. doi:10.1016/j.jchromb.2015.11.034
8. Veryser L, Taevernier L, Roche N, Blondeel P, De Spiegeleer B. Implementation of a single quad MS detector in high-throughput transdermal research of plant extracts. *J Pharm Biomed Anal*. 2015;115:594-602. doi:10.1016/j.jpba.2015.08.016
9. Rice SL, Hale RC. Parameters for ultra-performance liquid chromatographic/tandem mass spectrometric analysis of selected androgens versus estrogens in aqueous matrices. *Anal Chem*. 2009;81(16):6716-6724. doi:10.1021/ac900134m
10. Kumar N, Sangeetha D, Balakrishna P. Development and validation of a UPLC method for the determination of duloxetine hydrochloride residues on pharmaceutical manufacturing equipment surfaces. *Pharm Methods*. 2011;2(3):161-166. doi:10.4103/2229-4708.90355
11. ICH Harmonized Tripartite Guideline. *Validation of Analytical Procedures: Text and Methadology Q2(R1)*; 2005
12. Validation of Compendial Procedures. *General Information*. USP, 35:877-882.